DETERMINATION OF DENSITY OF AMOEBAE BY MEANS OF A STARCH DENSITY GRADIENT

by

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1. INTRODUCTION

In a paper published recently from this laboratory (HOLTER and LØVTRUP (5)) the localization of peptidase and proteinase in the amoeba Chaos chaos was investigated. The method employed was to centrifuge amoebae, cut them in two parts, light and heavy, and determine the specific enzyme activity (enzyme activity per μg reduced weight (RW)).

The reduced weight of an object equals the weight, W, of the object minus the weight of the volume of medium it displaces, $v \bullet \varphi_M$, where v is the volume of the object and φ_M the density of the medium. Thus

(I) $$RW = W - v \bullet \varphi_M(\varphi_0 - \varphi_M)$$

$\varphi_0$being the density of the object (LINDERSTRØM-LANG and HOLTER (6)). The results obtained (5) showed that the specific proteinase activity was higher in the heavy than in the light parts, while the reverse was found with respect to peptidase.

Since in view of previous results (see f. inst. the discussion by HOLTER (4)) it seemed unlikely that peptidase is centrifuged towards the light pole, it was thought that the higher specific peptidase activity in the light parts, when calculated on RW basis, might be due to the difference in density of the light and heavy parts, RW being very sensitive to changes in density of the object (equa-

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tion (1)). In order to check the latter possibility, it seemed desirable to recalculate the results on the enzyme distribution in the amoeba on a volume basis.

If RW of an object is known, then the volume can be calculated from equation (1), providing the density, φ_0, can also be measured. Density determinations on amoebae have been described by ZEUTHEN in his paper on reduced weight and volume of starving amoebae (9). The method employed consisted in a determination of the RW in two media of different density, viz. PRINGHEIM culture medium and a starch solution of $\varphi = 1.0121$. The accuracy of this method depends strictly on the density of the starch solution, a requirement which may be tedious to meet. If a polystyrene standard is used, its density must be known with accuracy. Since the commercial polystyrene from which the standards are made is inhomogenous, it is necessary to determine the density of the individual standards.

In the description of the method of volume and density determination (8), ZEUTHEN writes that "it will be necessary to substitute the soluble starch used by some other high molecular substance of more equimolecular constitution and less liable to undergo bacterial disintegration." The present author agrees with this, but although several other compounds have been tried, no other would fulfill the condition to form solutions of (relatively) high density, but low viscosity, being harmless to the amoebae. It was therefore necessary to continue the use of ZULKOWSKY starch.

To obtain as accurate density determinations as possible it was decided to try to use a starch gradient, since the accuracy of this method would be independent of small changes in the density of the starch solution. The following sections describe such an attempt and the results obtained by means of it.

2. METHOD

The density gradient method has been thoroughly described by LINDERSTRØM-LANG and LANZ (7). It might only be mentioned that the gradient tubes used in the present experiments were made smaller than those described earlier, partly because less accuracy was wanted, and partly to save starch and to facilitate the transfer of amoebae from the gradient. The radius of the bulbs was 4 cm, the distance between these 10 cm, and the inside diameter of the tube 2 cm.

The two gradient solutions were PRINGSHEIM culture medium and an approximately 20 per cent solution of ZULKOWSKY starch (SCHERING-KAHLBAUM A. G., Berlin), having a density of about 1.05. The starch solution was dialyzed 24 hours in cellophane bags, and boiled strongly to sterilize it before use.

The starch gradient obtained by mixing the two solutions is not as stable as a bromobenzene-kerosene gradient, partly because it may become infected by micro-organisms (moulds), and partly because after some time the starch may precipitate. This precipitation is especially harmful when it occurs on the glass standards (see later) thus changing their density. If sufficient care is taken, however, a gradient may last as long as one month. The starch gradient was standardized by means of small hollow glass beads, whose specific gravities were known from determinations in a bromobenzene-kerosene density gradient, standardized with KCl solutions (7). Five glass standards used had the following values of φ (all density determinations were made at 20 °C):

1.0040, 1.0143, 1.0222, 1.0229, and 1.0342.

Owing to the small difference in refraction index of glass and the bromobenzene-kerosene mixture, it was impossible to see sharply the contours of the glass bead. Since it was easy to see the air-filled space, the midpoint of this was used as reference to the position of the standards in both gradients. The values of φ given above refer to this level of the standards.

When the starch gradient had come into equilibrium the position of the standards did not change more than $\pm$ 0.1 mm, which is the accuracy of the cathetometer, with which the readings were taken. The steepness of the gradient is about 0.05 g/ml per 10 cm, or 0.0001 g/ml per 2 mm, which means that the change in the position of the standards corresponds to a change in φ of $\pm$ 0.00005 g/ml. When the density of an amoeba had to be determined it was transferred to the gradient tube by means of a braking pipette and blown out below the surface of the liquid. After 10-20 minutes the amoeba would come to rest, i.e. the change in position would be less than 0.1 mm in 10 minutes, and this "resting" level was read by the cathetometer. When the readings were finished, the amoeba was caught by means of a very long braking pipette.

The amoebae generally stayed about half an hour in the gradient, and a stay of this duration was apparently without any harmful effects. If an amoeba

was left overnight in the gradient, it would sink further down in the gradient and would ultimately come to rest on the bottom of the gradient tube.

3. EXPERIMENTS

3.1 . Density of light and heavy parts of centrifuged amoebae.

In this section will only be given a summary of the results obtained by measuring φ of the amoebae before and of the two parts after centrifugation[1] and cutting. These results are listed in table 1, where the letters A, *h*, and *l* refer to whole amoebae, heavy and light parts respectively.

The values of o of the unspecified amoebae are arranged according to size; the connection between the values of φ_A, φ_h, and φ_l for the same amoeba is unknown in these cases. One amoeba was lost during centrifugation. The two values of φ_h marked with an asterisk are surprisingly small, lower than the lowest value of the whole amoebae, which can only be explained by assuming that the corresponding heavy parts have expelled some of their heaviest material, which happens not too infrequently. It cannot be excluded that some of the other heavy parts have lost material, although it can be seen that in no other cases is φ_h smaller than φ_A. All values of have been used in calculating the average, since this value was to be used for correction in another series of centrifugation experiments, which had been performed under the same conditions, and where probably a similar loss of material had taken place. The influence of $\overline{\varphi_h}$ and $\overline{\varphi_l}$ (the bars indicate average values) upon the values for the distribution of proteolytic enzymes in centrifuged amoebae (HOLTER and LØVTRUP (5)) will be dealt with in the discussion.

A comparison of the values of φ_A in table 1 with the initial values obtained by ZEUTHEN (9) (specific gravities ranging from 1.017 to 1.025, corresponding to densities from 1.015 to 1.023) shows that the average values are of the same order of magnitude.

3.2. Volume determination of amoebae.

When the density and reduced weight of the amoebae are known, the volume is calculated from equation (1), rewritten in the following way:

[1] The centrifugation procedure was similar to that designated “strong” centrifugation in the paper on proteolytic enzymes (5).

Table 1.

Density of heavy and light parts of centrifuged amoebae

	amoeba 2	amoeba 9	unspecified amoebae	average	standard deviation
φ_A	1.0199	1.0163	1.0196, 1.0195, 1.0192, 1.0190, 1.0186, 1.0180, 1.0180, 1.0179	1.0186	0.0011
φ_h	1.0259	1.0233	1.0278, 1.0258, 1.0250, 1.0228, 1.0209, 1.0173*, 1.0162*	1.0228	0.0039
φ_l	1.0104	1.0105	1.0142, 1.0139, 1.0130, 1.0129, 1.0123, 1.0122, 1.0095	1.0121	0.0016

Table 2.

Comparision of volumes of amoeba parts and whole amoeba

	RW (μg)	φ (mg/μl)	v (μl × 10^3)	*RW* (μg)	φ (mg/μl)	v (μl × 10^3)	*RW* (μg)	φ (mg/μl)	v (μl × 10^3)	*RW* (μg)	φ (mg/μl)	v (μl × 10^3)
a	0.80	1.0161	45	1.20	1.0206	53.5	0.55	1.0135	36	0.755	1.0138	48
b	1.17	1.0158	66.5	0.96	1.0209	43	0.72	1.0138	46	0.55	1.0133	36
a + b	1.97	..	111.5	2.16	..	96.5	1.27	..	82	130.5	..	84
A	1.91	1.0157	109	2.14	1.0206	96	1.25	1.0138	80	129.5	1.0137	83.5

$$v = \frac{RW}{\varphi_A - 0.9982}$$

where the value of the density of water at 20° C has been inserted.

To obtain a control of the accuracy with which the volume can be measured by this method, the following experiment was made: An amoeba was weighed on the diver balance (ZEUTHEN (8)) and determined in the gradient tube, whereupon the amoeba was cut in two parts and RW and φ of these parts determined separately. We had to choose uncentrifuged amoebae for these experiments because centrifuged amoeba parts often will expel some of their material, which of course renders a control of the volume method impossible. The results obtained from such experiments are summarized in table 2, where *A* designates whole amoeba, *a* and *b* the two parts.

One experiment out of five has been discarded because the sum of the RW of the two parts was 0.12 μg smaller than the *RW* of the whole amoeba. Since this deviation was much greater than the error involved in *RW* determinations (standard deviation ~0.015 μg), the most reasonable explanation was that the amoeba had lost some material, probably due to the cutting.

The agreement between the volume of the whole amoeba and the parts is satisfactory and corresponds very well to the standard deviation of the volume determination (0.5–1 $\times 10^{-3}$ μl), which can be calculated if the standard deviation on determination of φ is taken to be 0.0001 and the standard deviation of *RW* determinations is 0.015 μg (see HOLTER and LØVTRUP (5)).

A comparison of the colorimetric volume determination of amoebae introduced by HOLTER (3) and the method here described shows that the accuracy is of the same order.

The small density differences between the whole amoebae and the parts may be solely experimental errors, but it should be mentioned that such changes in density also might arise as the result of the osmotic regulation of the amoebae. Transport of water through the cell membrane, whether active or not, sets a limit to the accuracy with which density, and accordingly volume determinations, can be made.

It should be emphasized that the present method cannot be employed for organisms without "active" osmoregulation, such as f. inst. eggs, unless the osmotic concentrations of the gradient solutions are carefully controlled.

4. DISCUSSION

From morphological studies (ANDRESEN (1)) it is known that when an amoeba is centrifuged most of the inclusions assumed to consist of "inactive" material, oil droplets and vacuoles of various kinds, are localized in the vicinity of the two poles. The total volume occupied by these inclusions is small compared with the volume of the cytoplasmic matrix (ANDRESEN and HOLTER (2)). Furthermore the proportion of inactive inclusions can be judged to be roughly the same in both parts of the amoeba. In first approximation, therefore, the total volume of the parts can be assumed to be proportional to the amount of "active cytoplasm" present (volume of "active cytoplasm" is total volume minus the volume of "inactive" material), so that two centrifuged parts, light and heavy, of equal volume also have equal amounts of "active cytoplasm". On the other hand, the ratio of RW of two such parts would be 0.57 $\left(\frac{l}{h}, \textit{ see later}\right)$.

It thus seems that the volume affords the best basis obtainable at present for the comparison of enzyme activities in amoebae parts. To convert the values of the comparison quotients (Q_{pe} = ratio of peptidase activity per μg RW of light and heavy parts, and correspondingly Q_{pr} for proteinase activity) obtained in (5), on a volume basis, it is sufficient to multiply these by the ratio $\frac{\overline{\varphi_l} - 0.9982}{\overline{\varphi_h} - 0.9982}$ (compare the equation, section 3.2). This latter value is seen from table 1 to be $\frac{0.0139}{0.0246} = 0.57$ (the standard error of this average ratio is 0.04). On a volume basis the values of the comparison quotients thus become Q'_{pe} = 1.76 • 0.57 = 1.00 and Q'_{pr} = 0.32 • 0.57 = 0.18 (see (5), footnote p. 59).

Even with proper regard to experimental errors and to the assumption made that two independent series of centrifugations and cuttings give the same values of $\overline{\varphi_l}$, and $\overline{\varphi_h}$, it may be safely concluded that centrifugation has little influence on the distribution of peptidase activity in amoebae, while the proteinase is associated with some cytoplasmic constituent which is concentrated in the "heavy" end of the amoebae, when these are subjected to a centrifugal force of ~1000 × g for about half an hour.

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SUMMARY

By means of a starch density gradient, density determinations have been performed on uncentrifuged amoebae, and heavy and light parts obtained by centrifugation. The results obtained have been used to recalculate the distribution of proteolytic enzymes in *Chaos chaos* on a volume basis. It has been shown how a combination of this method with determinations of reduced weight on the ZEUTHEN diver balance allows an estimation of the volume of the amoebae with an accuracy of 1×10^{-3} μl.

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